



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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<b>(54) Title:</b> <b>SPECIFIC DNA SEQUENCES OF A NEMATODE WHICH CAN BE USED FOR THE DIAGNOSIS OF INFECTION WITH THE NEMATODE</b>  <b>(57) Abstract</b>  <p>Method for the diagnosis of a worm infection, with which method DNA from the total DNA from the eggs of the nematode is amplified, in which method a DNA sequence comprising a species specific part of the 3' half of the <math>\beta</math>-tubulin gene is used as primer or in which method two DNA sequences of parts of the <math>\beta</math>-tubulin gene are used which parts are conserved in various nematodes as primers, which primers are located in the DNA sequence of the <math>\beta</math>-tubulin gene in such a manner that the amplification product has species specific length per nematode. Preferably a method for the diagnosis of infection with <i>H. contortus</i> using a DNA sequence comprising a species specific part of the 3' half of the <math>\beta</math>-tubulin gene of <i>H. contortus</i> as primer. The invention is also directed at a DNA sequence of a tubulin gene of <i>Haemonchus contortus</i> or a part thereof and preferably at a DNA sequence of the beta-tubulin gene of <i>Haemonchus contortus</i> or a part thereof. A method for the diagnosis of an infection with <i>Haemonchus contortus</i>, with which method the total DNA from the eggs of the nematode is applied to a (nylon) membrane and hybridised with a DNA probe being a sequence which comprises at least a part of one of the above mentioned sequences.</p>		

# + DESIGNATIONS OF "SU"

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SPECIFIC DNA SEQUENCES OF A NEMATODE WHICH CAN BE USED  
FOR THE DIAGNOSIS OF INFECTION WITH THE NEMATODE.

5           The present invention relates to a method for the diagnosis of  
a worm infection of nematodes in general and at kits for carrying out  
said diagnosis, in particular at the diagnosis of Haemonchus contortus.

10           Furthermore, the invention is also directed at specific DNA  
sequences of a tubulin gene of Haemonchus contortus and parts thereof, at  
insertions of the sequences in vectors and microorganisms and at the use  
of said sequences, inter alia for the diagnosis of infection with said  
nematode.

15           Parasitic helminths (worms) cause serious infectious diseases  
in humans and animals. In the case of livestock, infections arise at all  
ages and in virtually every farm.

20           The use of anthelmintics is widespread in veterinary medicine.  
In the Netherlands 35 million guilders were spent on anthelmintics in  
1987. In 1985 worldwide spending on anthelmintics was US\$ 540 million.  
The total costs of parasitic worm infections in cattle (anthelmintics,  
loss of production, etc.) are estimated to be 100 to 200 million guilders  
per year in the Netherlands (thesis by H.W. Ploeger 1988, Agricultural  
University of Wageningen).

25           Little is known about the molecular biology of the mechanism of  
action of the various control agents. Three groups of broad spectrum  
anthelmintics are known, each of which have their own mechanism of  
action. These are

30           i)       benzimidazoles, which probably act via binding to tubulin and  
consequently inhibit transport within the cell and cell division (Lacey,  
E., 1988, The role of the cytoskeletal protein, tubulin, in the mode of  
action and mechanism of drug resistance to benzimidazoles, Int. J.  
Parasitol. 18, 885-936).

          ii)       levamisole and morantel, ganglion stimulants which lead to  
paralysis of the parasite, and

35           iii)      ivermectins, which intensify the inhibitory effect of gamma-  
aminobutyric acid (GABA). In nematodes GABA is the most important  
neurotransmitter for inhibitory signals from interneurons to motor  
neurons (Barragry, T., 1984a, Anthelmintics - A review, N.Z. Vet. J. 32,  
161-164; Barragry, T., 1984b, Anthelmintics- review Part II, N.Z. Vet. J.  
32, 191-199).

Of these three, the benzimidazoles are the most widely used.

Using molecular biological techniques, we have studied one of the most harmful gastrointestinal parasites in sheep: Haemonchus contortus. This occurs worldwide, causes haemonchosis and is responsible for losses of 50 million guilders per year in sheepfarming in the Netherlands.

With a view to effective control of this harmful organism, a sensitive and reliable method for the detection of this worm is indispensable. Parasitic worm infections in sheep are usually mixed infections and a method is therefore needed which is able to differentiate between H. contortus and the other parasitic worms present. The methods which are currently used for the diagnosis of H. contortus are:

- 1) the detection of the specific symptoms of the disease in sheep, and
- 2) differentiation of the larval culture from faeces of the sheep.

A disadvantage of 1) lies in the fact that clinical diagnosis of an infection with parasitic intestinal worms is difficult to make because the symptoms of the disease cannot always be differentiated from those of other diseases or poor nutrition. In the case of H. contortus the external symptoms (inter alia anaemia and oedema) are only detectable when there is a severe degree of infection, whereas damage (loss in weight, wool loss, milk loss) is already caused by an infection without these external symptoms of disease.

A disadvantage of method (2) lies in the fact that the eggs of various nematodes in the faeces of infected animals appear to be very similar to one another, as a result of which in the case of eggs it is not possible to make a diagnosis of the worm species. For this purpose, a larval culture must first be carried out, which takes about one week. In practice, this method is little used (Janssens, P.G., Vercruysse, J. and Jansen, J., editors: Wormen en wormziekten bij mens en dier (Worms and worm diseases in humans and animals) (1989) Samsom Stafleu, Alphen aan de Rijn/Brussels).

A new, rapid and species-specific test is therefore needed to be able to diagnose infection with H. contortus. In the case of other nematodes there are a number of tests which are used, such as:

- a) the polymerase chain reaction (PCR), and
- b) the dot-blot assay.

The dot-blot assay is very sensitive and rapidly yields

results, such as, for example, in the diagnosis of the human nematode Brugia, which causes filariasis (McReynolds, L.A., deSimone, S.M., Williams, S.A. (1986) Cloning and comparison of repeated DNA sequences from the human filarial parasite Brugia pahangi, Proc. Natl. Acad. Sci. USA 83, 797-801). In this test, total DNA from the eggs is applied to a nylon membrane and hybridised with the specific DNA probe.

In the case of PCR, specific DNA sequences can be used as primer, by which means a fragment of the specific gene is amplified. This can be rendered visible by analysing the reaction mixture using an agarose gel, or by coupling a (fluorescent) label to the primer (De Bruijn, M.H.L., (1988) Diagnostic DNA amplification: no respite for the elusive parasite, Parasitol. Today, 4:293-295). To date, no Haemonchus contortus DNA is known which is specific for the species and therefore these tests are not possible for the diagnosis of infection with H. contortus. Surprisingly, it has been found that DNA coding for tubulin is species-specific for H. contortus.

The present invention relates to a DNA sequence of a tubulin gene of H. contortus or a part thereof. The DNA sequence of the tubulin gene or a part thereof comprises the alpha-tubulin gene of H. contortus or a part thereof or the beta-tubulin gene of H. contortus or a part thereof. ( In figure 1 the DNA and aminoacid sequence of the  $\alpha$ -tubulin clone, determined as described further on in the example are given. The numbering of the aminoacids in the  $\alpha$ -tubulin gene is from 395-425) ( In figure 2 DNA and amino acid sequence of the  $\beta$ -tubulin clone determined as described further on in the example, are illustrated. The numbering of the DNA bases is given above the DNA sequence, that of the amino acids is given below the DNA sequence. The clone contains the 3' half of the gene and the numbering of the amino acids in the  $\beta$ -tubulin gene is 223-448. The polyadenylation signal (bases 1538-1543) is underlined. The site of the oligonucleotide primers is illustrated with a dashed line above the DNA sequence:  $\beta$ -1, bases 1-19 according to the strand illustrated here;  $\beta$ -2 bases 1311-1331 according to the complementary strand).

It was known from study previously published DNA-sequences of  $\beta$ -tubulin genes of other organisms that  $\beta$ -tubulin genes are strongly conserved [(Cleveland, D.W. and Sullivan, K.F. (1985) Molecular Biology and genetics of tubulin. Ann. Rev. Biochem., 54; 331-365), (Little, M. and Seehaus, T. (1988) Comparative analysis of tubulin sequences. Comp. Biochem. Physiol., 90B; 655-670), (MacRae, T.H. and Langdon, C.M. (1989) Tubulin synthesis, structure and function: what are the relationships?

Biochem. Cell Biol., 67: 770-789)].

Furthermore, it was apparent from previously published DNA-sequences of a number of nematodes (also of  $\beta$ -tubulin, see Driscoll et al. The Journal of Biology, 1989, volume 109, pages 2993-3003) that the  
5 known nematode DNA sequences mostly resemble higher eucaryotic tubulin sequences. Various  $\beta$ -tubulin genes occur in most higher eucaryotes. From what is presently known there appears to be more difference between the different types (genes) of  $\beta$ -tubulin within one animal species than  
10 between the same type among different animal species. For example: the degree of conservation between tubulin type  $\beta$ -4 and  $\beta$ -5 of the human is 91,2 %, whereas conservation between type  $\beta$ -4 of the human and type  $\beta$ -4 of the chicken is 99,3 %.

Considering the state of the art as described above it is therefore surprising that DNA of  $\beta$ -tubulin is species specific for H. contortus.  
15

Fragments of a DNA-sequence according to the invention can be used as oligonucleotide probes for specifically determining H. contortus especially in mixed infections. Preferably the PCR method is used for this. Probes comprising a part of the sequence according to the invention  
20 react specifically with H. contortus, whereby a probe comprising a part of the  $\beta$ -tubulin sequence is especially suited for use in specifically determining H. contortus in this manner.

Upon studying the conservation of DNA of tubulin genes it was found that the location of the introns is generally conserved. Introns  
25 generally occur in the 5' half of the tubulin gene. In the article of Driscoll et al. already cited it was demonstrated that introns also occur in the 3' half of the 3  $\beta$ -tubulin genes of the nematode C. elegans. These introns can be distinguished from the introns of the  $\beta$ -tubulin genes of other higher eucaryotes.

It has now been found that the DNA-sequence of the 3' half of the  $\beta$ -tubulin gene of H. contortus also contains introns. The 3' half of  $\beta$ -tubulin of H. contortus has been found to contain a lot more introns  
30 than the 3' half of  $\beta$ -tubulin of C. elegans and the introns also differ in length from the introns of C. elegans. The location and length of introns in the 3' half of the  $\beta$ -tubulin gene appears not to be conserved  
35 in nematodes.

Further research of  $\beta$ -tubulin genes of other nematodes further to the above mentioned discovery have revealed that there are other nematodes beside H. contortus and C. elegans which also contain non

conserved introns in the 3' half of their  $\beta$ -tubulin genes.

The subject invention is therefore in general directed at the use of a DNA sequence of a specific nematode as oligonucleotide probe, which probe comprises a part of the DNA sequence of the 3' half of a  $\beta$ -tubulin gene, for specifically determining the specific nematode. The subject invention is therefore not restricted to a method for specifically detecting H. contortus in which method a DNA sequence is used as oligonucleotide probe comprising a part of the 3' half of the  $\beta$ -tubulin gene of H. contortus. The detection of H. contortus in this manner serves as an example of a method for the diagnosis of an infection with a certain nematode according to the subject invention.

Another suitable example of a nematode which can specifically be detected using an oligonucleotide probe comprising a part of the DNA sequence of the 3' half of the  $\beta$ -tubulin gene is Trichostrongylus colubriformis.

The presence of differences in the 3' half of  $\beta$ -tubulin genes of nematodes when compared with each other made a species specific PCR possible. Parts of a DNA sequence comprising a  $\beta$ -tubulin gene can be used as oligonucleotide probes for specifically detecting a nematode with the aid of the PCR in mixed infections.

A particular embodiment of the method that has been described above comprises the use of parts of the  $\beta$ -tubulin gene as primers, which primers are conserved in various nematodes, whereby two sequences are selected as primers which are located in such a manner that the DNA sequence which is amplified from the total DNA of the nematode via PCR, the amplification product, has a species specific length per nematode, because the DNA sequence which is located between the primers is species specific. It is preferable to amplify DNA from the DNA sequence comprising a part of the 3' half of the  $\beta$ -tubulin gene, as the DNA sequence of the 3' half has been found to differ in intron length per nematode.

Two  $\beta$ -tubulin oligonucleotides,  $\beta$ -1 and  $\beta$ -2 were made on the basis of a DNA sequence of the  $\beta$ -tubulin gene of H. contortus as determined by the inventors and illustrated in figure 2, in order to be able to measure the difference in introns among various nematode species. The oligonucleotides namely contain DNA sequences coding for amino acid sequences supposed to be conserved in  $\beta$ -tubulin genes of all higher eucaryotes. Oligonucleotide  $\beta$ -1 comprises amino acids 1-7 of the  $\beta$ -tubulin gene of H. contortus and oligonucleotide  $\beta$ -2 comprises amino

acids 198-204. The DNA sequence of  $\beta$ -1 is: 5' ACGGATCTCAACCACCTTG 3' and the sequence of  $\beta$ -2 on the complementary strand is 5' CTGGTACTGCTGGTATTCGGA 3'.

5 The PCR carried out with these oligonucleotides always gave a positive reaction to the presence of H. contortus DNA also in mixed infections. With this PCR a DNA fragment of approximately 1300 bases was generated. This PCR always gave a positive result when H. contortus DNA was present and then the fragment of 1300 bases was found with the  $\beta$ -1 and  $\beta$ -2 primers. The PCR always gave a negative reaction when no fragment  
10 of 1300 bases was present and no H. contortus DNA was present.

The PCR with the  $\beta$ -1 and  $\beta$ -2 primers was also tested on DNA of other prevalent sheep parasites. A positive reaction was also found with Trychostrongylus colubriformis also in mixed infections, however the DNA fragment was smaller approximately 900 bases and clearly discernable from  
15 the H. contortus fragment.

The PCR with the  $\beta$ -1 and  $\beta$ -2 primers was also tested on DNA of Ostertagia circumcincta, Nippostrongylus brasiliensis, Trichinella spiralis and Trichinella nativa. No reaction was found not even with so called degenerate primers, which take into account the degeneracy of the  
20 genetic code. These were primers  $\beta$ -7 (amino acids 1-6) with DNA sequence: 5' (CTA)G(GC)(GTAC)GA(CT)(CT)T(GCAT)AA(CT)CA(CT)(CT)T 3' and  $\beta$ -8 (amino acids 199-205) on the complementary strand with DNA sequence: 5' TC(CT)TGGTA(TC)TG(CT)TG(GA)TA(CT)TC 3' (figure 2). The PCR with the  $\beta$ -7 and  $\beta$ -8 primers was positive with DNA of H. contortus and T. colubriformis giving the DNA fragments that were described previously.  
25

As the used primers correspond to a DNA sequence coding for a very conserved region of the  $\beta$ -tubulin molecule it appears probable that the  $\beta$ -tubulin gene(s) of the nematode species leading to a negative result with these primers using PCR contain an intron in the sequence of  
30 the primers. This could prevent the primer from binding and could prevent any reaction taking place. A change in the amino acid sequence in the region of the primers could also be a possible explanation for the above mentioned result but does not seem probable considering the conservation of the amino acid sequence in this region.

35 By selecting the primers in a different region of the 3' half of the  $\beta$ -tubulin gene a species specific PCR can also be obtained for the nematodes giving a negative result with the primers  $\beta$ -1,  $\beta$ -2,  $\beta$ -7 and  $\beta$ -8.

The subject invention is therefore also directed at a method



for the diagnosis of a worm infection, with which method the total DNA from the eggs from the nematode is amplified preferably via PCR, which method is characterized in the use of two parts of the coding sequence of the  $\beta$ -tubulin gene which are conserved in various nematodes as primers, which primers are located in the DNA sequence of the  $\beta$ -tubulin gene in such a manner that the amplification product has a species specific length per nematode.

The beta-tubulin probes comprising the complete 2,2 kb  $\beta$ -tubulin clone of H. contortus (pEMBLaB, deposited at the CBS in Baarn under Nr. CBS 335.90) or the 1,3 kb  $\beta$ -1/ $\beta$ -2 PCR fragment always show a positive reaction to the presence of H. contortus, even in the case of a mixed infection. The beta-tubulin probes of H. contortus were also tested on the DNA of other frequently occurring sheep parasites and this reaction was found to be much less pronounced than with its own species. Yet specific bands were obtained with these large probes which indicates conservation. The weaker reaction could be caused by introns in conserved regions or small species specific DNA sequences within these large probes. The species which were tested are, inter alia: Cooperia circumcincta, Nematodirus battus, Nippostrongylus brasiliensis, Ostertagia circumcincta, Trichostrongylus colubriformis, Trichostrongylus vitrinus, Trichinella spiralis and Trichinella nativa.

By selecting a specific sequence from a DNA sequence according to the invention of for example, at least 10 nucleotides from a H. contortus specific intron or the DNA sequence coding for amino acids 332-335, which under specific conditions reacts solely with H. contortus, it is possible to establish a test by means of which it is possible, in the case of a mixed infection with nematodes, to diagnose infection with H. contortus from the DNA of the eggs.

Examples of tests which can be used are those which are used in the case of other nematodes:

- a) the polymerase chain reaction (PCR), and
- b) the dot-blot assay.

The present invention also relates to a method for the diagnosis of a worm infection, in which method the total DNA from the eggs from the nematode is applied to a nylon membrane and hybridised with a DNA probe, which method is characterised in that a diagnosis of infection with Haemonchus contortus is made, using a sequence which corresponds to a specific part of a tubulin gene of H. contortus as oligonucleotide probe. A preferred method is a method in which specific

DNA fragments from the DNA of the eggs are amplified, for example by means of PCR, and are rendered visible. The diagnosis of an infection with H. contortus can be made because the amplification of specific fragments is successful when H. contortus DNA is present in the egg sample and sequences are used, such as a primer which corresponds to at least a conserved part of a tubulin gene of H. contortus, whereby said sequences are localised in the DNA sequence of the  $\beta$ -tubulin gene in such a manner that the amplification product has a species specific length.

The invention is also directed at a kit for carrying out the diagnosis of a worm infection which kit comprises at least a species specific part of the 3' half of the  $\beta$ -tubulin gene of the nematode to be detected as oligonucleotide probe and is also provided with other means and materials which are apparent to an expert.

The invention is further directed at a kit for carrying out a method for the diagnosis of infection with H. contortus, which kit comprises at least a part of the DNA sequence according to the invention and is also provided with other means and materials apparent to an expert. Such a kit shall preferably comprise a part of the 3' half of the  $\beta$ -tubulin gene of H. contortus.

The invention is further directed at a kit for carrying a method for the diagnosis of infection with H. contortus, which kit comprises at least primers comprising coding DNA sequences of the  $\beta$ -tubulin gene which are conserved in various nematodes, which primers are located in such a manner in the DNA sequence of the  $\beta$ -tubulin gene of H. contortus that the amplification product has a species specific length. Such a kit shall preferably comprise primers giving a part of the 3' half of the  $\beta$ -tubulin gene of H. contortus as amplification product. Such a kit will comprise for example the  $\beta$ -1 and  $\beta$ -2 primers and/or the  $\beta$ -7 and  $\beta$ -8 primers. Furthermore such a kit is also provided with the means and materials apparent for an expert.

The sequences according to the invention can also be used if they are incorporated in a vector, an expression vector being preferred. The invention relates to vectors of this type and also to recombinant microorganisms which comprise a DNA sequence according to the invention.

The invention also relates to a peptide which comprises at least one fragment of the amino acid sequence which is coded by a DNA sequence according to the invention.

Peptides of, for example, at least 15 amino acids are suitable as antigen, for example for the generation of specific antiserum, or

monoclonal antibodies against H. contortus. The invention relates to these antibodies. Antibodies of this type can also be used for the diagnosis of an infection with Haemonchus contortus. The invention relates to this use and to the use of the antibodies in a kit for the

5     diagnosis of infection with H. contortus.

EXAMPLE

Construction of the gene libraries of H. contortus and analysis of the tubulin clones

Genomic DNA was made from L3 larvae using the method of Coulson et al. (Coulson, A., Sulston, J., Brenner, S. and Karn, J. (1986) Toward a physical map of the genome of the nematode Caenorhabditis elegans, Proc. Natl. Acad. Sci. USA 83, 7821-7825). The size of the DNA fragments to be cloned was 20 kb. In order to obtain arbitrary fragments of this size a partial digestion was carried out with a restriction enzyme that

10     cuts frequently in genomic DNA. To this end, the concentration of the enzyme was chosen as described in Maniatis et al. (Maniatis, T., Fritsch, T. and Sambrook, J. (1982) Molecular cloning. A laboratory manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., USA). These fragments

15     were isolated and ligated in the DNA of the bacteriophage arms of EMBL3 using the method of Frischauf et al. (Frischauf, A.M., Lehrach, H., Poustka, A., Murray, N. (1983) Lambda replacement vectors carrying polylinker sequences, J. Mol. Biol. 170, 827-841). This DNA construct was then packaged in bacteriophage protein envelope and used to infect the bacterial strain Le 392. The infected bacteria were plated out with non-

20     infected bacteria and after incubation at 37°C a plaque was visible at the location where infected bacteria were present (Frischauf, A.M., Lehrach, H., Poustka, A. and Murray, N. (1983) Lambda replacement vectors carrying polylinker sequences, J. Mol. Biol. 170, 827-841). The plaques were then screened according to the method of Maniatis et al. (Maniatis, T., Fritsch, T. and Sambrook, J. (1982) Molecular cloning. A laboratory

25     manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., USA). The tubulin probes used to isolate the alpha-tubulin DNA-containing clones were chicken alpha-tubulin [as described in Valenzuela et al. (Valenzuela, P., Quiroga, M., Zaldivar, J., Rutter, W.J., Kirshner, M.W. and Cleveland, D.W. (1981) Nucleotide and corresponding amino acid sequences encoded by alpha and beta tubulin mRNAs. Nature 289, 650-655)]

30     and Drosophila hydei alpha-tubulin [a gift from Dr. R. Brand of the Catholic University of Nijmegen (Michiels, F., Falkenburg, D., Muller, A.M., Hinz, U., Otto, U., Bellmann, R., Glazer, K.H., Brand, R.,

35     ]

Bialojan, S. and Renkawitz-Pohl, R. (1987) Testis-specific beta2 tubulins are identical in Drosophila melanogaster and D. hydei but differ from the ubiquitous beta1 tubulin)].

5 The beta-tubulin probes used to isolate the beta-tubulin DNA-containing clones were a beta-tubulin clone of Caenorhabditis elegans, Ben-1 [which is described in Driscoll et al. (Driscoll, M., Dean, E., Reilly, E., Bergholz, E. and Chalfie, M. (1989) Genetic and molecular analysis of a Caenorhabditis elegans beta tubulin that conveys benzimidazole sensitivity. J. Cell Biol. 109, 2993-3003)] and a beta-  
10 tubulin clone of Drosophila hydei pTB5 [a gift from Dr. R. Brand of the Catholic University of Nijmegen (Michiels, F., Falkenburg, D., Muller, A.M., Hinz, U., Otto, U., Bellmann, R., Glazer, K.H., Brand, R., Bialojan, S. and Renkawitz-Pohl, R. (1987) Testis-specific beta2 tubulins are identical in Drosophila melanogaster and D. hydei but differ from the  
15 ubiquitous beta1 tubulin)].

Of the clones obtained in this way, the clone pEMBL8cA (deposited with the CBS in Baarn under No. CBS 335.90), for alpha-tubulin, and the clone pEMBL7aB (deposited with the CBS in Baarn under No. CBS 334.90), for beta-tubulin, were further characterised. Subclones  
20 thereof, which contain DNA fragments coding for protein, were constructed in the vectors Bluescript and M13. The DNA of these subclones was sequenced using the dideoxy method of Sanger et al. (Sanger, F., Nicklen, S. and Coulson, A.R. (1977) DNA sequencing with chain terminating inhibitors, Proc. Natl. Acad. Sci. USA 74, 5463-5467), as modified in the  
25 T7 sequencing kit from Pharmacia and the M13 sequencing kit from Amersham. Standard primers, which are supplied with the kits, were used. The analysis and comparison of the sequences thus obtained was carried out using the Microgenie (Beckman) and PC/Gene (Genofit, Geneva) computer programs. The sequences determined for these clones are shown in Figures  
30 1 and 2 respectively.

The PCR specific for H. contortus and T. colubriformis.

The oligonucleotides B-1 and B-2 as described above were made in order to carry out a species specific PCR for DNA of H. contortus and T. colubriformis. The B-7 and B-8 primers were made in order to obtain a  
35 species specific PCR for the species O. circumcincta, T. spiralis and T. nativa. The PCR method was used according to Ehrlich et al. (Saiki, R.K., (1989) in: PCR technology, principles and applications for DNA amplification, Erlich, H.A. (ed.), Stockton Press, New York, London, pp. 7-16). The reaction conditions used in the present invention, which were

used to amplify the DNA, are: PCR with  $\beta$ -1 and  $\beta$ -2 primers with a magnesium concentration of 1,5 mM an annealing temperature of 61°C for 30 seconds, elongation at 72°C for 2 minutes and denaturing at 95°C for 30 seconds. This method amplifies the beta-tubulin of H. contortus and T. colubriformis from the genomic DNA, which can be rendered visible by electrophoresis in an agarose gel, whereby DNA of H. contortus gives a visible band of 1,3 kb and DNA of T. colubriformis gives a visible band of 0,9 kb. This method does not work with Ostertagia, N. brasiliensis, T. spiralis and T. nativa, which makes it clear that this PCR method with the abovementioned primers is specific for Haemonchus contortus and Trichostrongylus colubriformis.

The PCR conditions with the  $\beta$ -7 and  $\beta$ -8 primers were: 5 cycli as follows. An annealing temperature of 37°C for 1 minute, a slow rise in 5 minutes from 37°C to the elongation temperature of 72°C, 2 minutes at 72°C, 1 minute at 95°C. Subsequently 20 cycli of 1 minute at 55°C, 2 minutes at 72°C and 1 minute at 95°C. The magnesium concentration was 1,5 mM. This PCR visualized a positive reaction with H. contortus DNA and was negative with O. circumcincta, T. spiralis and T. nativa.

CLAIMS

1. DNA sequence of a tubulin gene of Haemonchus contortus or a part thereof.
2. DNA sequence of the beta-tubulin gene of Haemonchus  
5 contortus or a part thereof.
3. DNA sequence according to Claim 2, characterised in that this comprises the nucleotide sequence according to Fig. 2 or a part thereof.
4. DNA sequence according to Claim 3, characterised in that  
10 this is obtainable from clone pEMBL7aB (deposited with the CBS in Baarn under No. CBS 334.90).
5. DNA sequence according to Claim 1, characterised in that this comprises the nucleotide sequence according to Fig. 1 or a part thereof.
6. DNA sequence according to Claim 5, characterised in that  
15 this is obtainable from clone pEMBL8cA (deposited with the CBS in Baarn under No. CBS 335.90).
7. Method for the diagnosis of a worm infection, with which method the total DNA from the eggs of the nematode is applied to a  
20 (nylon) membrane and hybridised with a DNA probe, characterised in that a diagnosis of infection with Haemonchus contortus is made, the DNA probe used being a species specific sequence which comprises at least a part of a sequence according to one of the preceding claims.
8. Method for the diagnosis of a worm infection, with which  
25 method DNA from the total DNA from the eggs of the nematode is amplified, characterised in that a diagnosis of infection with Haemonchus contortus is made the primers used being DNA sequences which comprise at least a part of a DNA sequence according to one of Claims 1-6.
9. Method for the diagnosis of a worm infection, with which  
30 method DNA from the total DNA from the eggs of the nematode is amplified, characterised in that DNA sequences comprising species specific parts of the 3' half of the  $\beta$ -tubulin gene are used as primers.
10. Method for the diagnosis of a worm infection according to  
claim 9, characterised in that the diagnosis of infection with H.  
35 contortus is carried out using DNA sequences comprising species specific parts of the 3' half of the  $\beta$ -tubulin gene of H. contortus as primers.
11. Method for the diagnosis of a worm infection, with which method DNA from the total DNA from the eggs of the nematode is amplified, characterised in that two DNA sequences of the  $\beta$ -tubulin gene are used

which are conserved in various nematodes as primers, which primers are located in the DNA sequence of the  $\beta$ -tubulin gene in such a manner that the amplification product has species specific length per nematode.

12. A method according to claim 11, characterised in that  $\beta$ -1 and  $\beta$ -2 are used as primers, whereby  $\beta$ -1 has the sequence 5' ACGGATCTCAACGACCTTG 3' and  $\beta$ -2 has the sequence 5' CTGGTACTGCTGGTATTCGGA 3' on the complementary strand, or primers  $\beta$ -7 and  $\beta$ -8 are used whereby  $\beta$ -7 comprises the amino acids 1-6 of  $\beta$ -tubulin of H. contortus with DNA sequence: 5' (CTA)G(GC)(GTAC)GA(CT)(CT)T(GCAT)AA(CT)CA(CT)(CT)T 3' and  $\beta$ -8 comprises the amino acids 199-205 of  $\beta$ -tubulin of H. contortus with DNA sequence : 5' TC(CT)TGGTA(TC)TG(CT)TG(GA)TA(CT)TC 3' on the complementary strand (figure 2).

13. A method according to claim 12, characterised in that a diagnosis of infection is carried out which is specific for H. contortus when the amplification product has a length of 1,3 kb.

14. A method according to claim 12, characterised in that a diagnosis of infection is carried out which is specific for Trichostrongylus colubriformis when the amplification product has a length of 0,9 kb.

15. Peptide which comprises at least one fragment of the amino acid sequence which is encoded by a DNA sequence according to one of Claims 1-6.

16. Vector which comprises at least one DNA sequence according to one of Claims 1-6.

17. Vector according to Claim 16, characterised in that this is an expression vector which can express the DNA sequence according to one of Claims 1-6.

18. Recombinant microorganism which comprises a DNA sequence according to one of Claims 1-6 or a vector according to Claim 16 or 17.

19. Antibody which is generated against a peptide according to Claim 15.

20. Use of an antibody according to Claim 19 for diagnosing an infection with Haemonchus contortus.

21. Vaccine for protection against or for combating Haemonchus contortus, which comprises a peptide according to Claim 15 or a vector according to Claim 17 or a recombinant microorganism according to Claim 18.

22. Vaccine for combating Haemonchus contortus, which comprises an antibody according to Claim 19.

23. Kit for carrying out a diagnosis of infection with Haemonchus contortus, which comprises an antibody according to Claim 19.

24. Kit for carrying out a diagnosis of a worm infection which kit comprises oligonucleotide probes comprising a species specific part  
5 of the 3' half of the  $\beta$ -tubulin gene of the nematode to be detected.

25. Kit for carrying out the diagnosis of infection with H. contortus, which kit comprises at least a part of the DNA sequence according to one of the claims 1-6.

26. Kit for carrying out a diagnosis of infection with H. contortus, which kit comprises at least primers comprising coding DNA  
10 sequences of the  $\beta$ -tubulin gene which are conserved in various nematodes, which primers are located in such a manner in the DNA sequence of the  $\beta$ -tubulin gene of H. contortus that the amplification product has a species specific length.

27. Kit according to claim 25 or 26, which kit comprises a part  
15 of the DNA sequence of the 3' half of the  $\beta$ -tubulin gene of H. contortus.

28. Kit according to claim 27 which kit comprises the primers  $\beta$ -1 and  $\beta$ -2 and/or the primers  $\beta$ -7 and  $\beta$ -8.



Fig. 1

10 20 30 40 50  
TTTGCATGCT GTCCAACACG ACGCGATCGC TGAAGCTTGG GCTCGTTTGG ATATAAG

60 70 80 90 100  
TTT GAC CTT ATG TAT GCC AAG CGT GCA TTC GTC CAC TG GTGAG  
Phe Asp Leu Met Tyr Ala Lys Arg Ala Phe Val His Trp

10

110 120 130 140 150  
TGTTCCTTTCG ACATCATCTT TTTCAATTGC AGTTGTTCTG CATATACAAC

160 170 180 190 200  
ATTTTATGAA AGTCAGATAT ACTGTTTCAG G TAT GTC GGA GAG GGA ATG GAG  
Tyr Val Gly Glu Gly Met Glu

20

210 220 230 240 250  
GAA GGA GAG TTC AGT GAG GNA C GTGAAGA TCTCCCCGGG CTGCAGGAAT  
Glu Gly Glu Phe Ser Glu

260 270  
TCGATATCAA GCTTATCGAT ACCGT

Fig. 2 (1)

primer  $\beta$ -7

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primer  $\beta$ -1

-----

ACG GAT CTC AAC CAC CTT G G TAATTGTTAT TACACTTACT AAAGTATACT  
Thr Asp Leu Asn His Leu

50

TAGACCTTTT TTCATGCTGA AAATGTGCAA TTGAAG TG TCT GTC ACA ATG TCT  
Val Ser Val Thr Met Ser

100

10

140

GGT GTC ACG ACC TGC CTT CGA TTC CCT GGA CAG CTG AAT GCT GAT  
Gly Val Thr Thr Cys Leu Arg Phe Pro Gly Gln Leu Asn Ala Asp

20

190

CTT CGC AAG TTA GCC GTG AAC ATG GTT CCA TTC CCT CGT CTT CAC  
Leu Arg Lys Leu Ala Val Asn Met Val Pro Phe Pro Arg Leu His

30

40

230

TTC TTC ATG CCC GGT TTT GCT CCA CTG TCT GCA AAG GGT GCT CAA  
Phe Phe Met Pro Gly Phe Ala Pro Leu Ser Ala Lys Gly Ala Gln

50

280

GCA TAT CGC GCT TCG ACA GTT GCT GAG CTT ACA CAG CAA GTA  
Ala Tyr Arg Ala Ser Thr Val Ala Glu Leu Thr Gln Gln

60

70

3/5

2(2)

330

CGCCCATAC TCTTTATCAG CATCAGTGAT TCAAAATTAC CAGCCAGCGC ATTTAG

380

ATG TTC GAT GCA AAG AAC ATG ATG GCT GCC TGT GAT CCT CGC CAT  
Met Phe Asp Ala Lys Asn Met Met Ala Ala Cys Cys Pro Arg His

80

420

GGA CGT TAT CTT ACG GTC GCT GCT ATG TTC CGT GGT CGT ATG AGC  
Gly Arg Tyr Leu Thr Val Ala Ala Met Phe Arg Gly Arg Asn Ser

90

100

480

ATG CGA GTGAGTAT TTTTAAACGT GTTTTTTCAT CAAGTAAATG ATGAATACTG  
Met Arg

530

GTGGGACTGG AAATGAGTCA GAAGTAGTGT TCTAATGTGA GCTATTCACA

580

AAACTTGTA TCTCACTGTG GGCCAAGACT TCTACATGGC AAGCCAGATG

630

TTTTATAAAT ACAGTGCTAT GAGTAGCAAC CATATATAGT TTCTATAAGA

680

TCGCTCTAAG ATATGAATGC CACCGGTATA ACTAGTTACT GGTTACTTAT

730

GCCATGAAGG AGTAGTAATT GATGCAGGAT GTCATCTGCT ATAATCTAAT

780

CGCAATAATA TTGTAGCTTT GAGAGACCTG CAGCATCCTC GCTACCGTAG

830

CCCGCATAGT TTTTAAAGCG AAACGGGTAG GCTATTGTGG TTTTTTGGGG

2(3)

880

GCATTTTTTT CTGAAATGCT GCAGTCAATT GTAAAACCGC GATGTGATTG

930

TGTGATCGGT TCATTGCAGT ATTTTGTATC GTAGAGATAA GAAAATATCA

980

TGTGGAAGGT CAACTGCTTC ATTTTCAG GAA GTA GAT GAT CAG ATG ATG TCC

Glu Val Asp Asp Gln Met Met Ser

110

1020

GTG CAG AAC AAG AAC TCA TCA TAT TTC GTG GAA TGG ATT CCA AAC  
Val Gln Asn Lys Asn Ser Ser Tyr Phe Val Glu Trp Ile Pro Asn

120

1070

AAC GTT AAG ACT GCC GTT TGT GAC ATT CCT CCT CGT GGA CTG AAA  
Asn Val Lys Thr Ala Val Cys Asp Ile Pro Pro Arg Gly Leu Lys

130

140

1110

ATG GCG GCT ACC TTC GTT GGT AAC TCG ACT GCT ATC CAG GAG CTG  
Met Ala Ala Thr Phe Val Gly Asn Ser Thr Ala Ile Gln Glu Leu

150

1160

TTC AAG CGT ATT TCG GAG CAA TTC ACT G GTTTGT TTTGATTTAT  
Phe Lys Arg Ile Ser Glu Gln Phe Thr

160

1210

GAGCTGTCGT ACATGAGTGC GTCTTGATTC CAACACTCTT CACCGCTTCA G CC

Ala

1250

ATG TTC CGA CGC AAA GCT TTC CTT CAT TGG TAC ACT GGT GAG GGT  
Met Phe Arg Arg Lys Ala Phe Leu His Trp Tyr Thr Gly Glu Gly

170

180

5/5

2(4)

1300

ATG GAC GAA ATG GAG TTC ACA GAA GCT GAG TCG AAC ATG AAT GAC  
Met Asp Glu Met Glu Phe Thr Glu Ala Glu Ser Asn Met Asn Asp

190

primer  $\beta$ -8primer  $\beta$ -2

1350

CTT ATC TCC GAA TAC CAG CAG TAC CAG GTCCGTATTA TCTTATTCCA  
Leu Ile Ser Glu Tyr Gln Gln Tyr Gln

200

1400

GCTATGTCTT AGCGTGATCG TAGACGTTTA GTCTTTAATA TTTCCAG GAA GCT  
Glu Ala

1440

ACC GCT GAC GAT ATG GGC GAT CTC GAT GCA GAA GGT GGA GAA GAG  
Thr Ala Asp Asp Met Gly Asp Leu Asp Ala Glu Gly Gly Glu Glu

210

220

1500

GCA TAT CCC GAG GAG TAA TGAT CCACAAAGTT GTGCTCTTTT TCCTGTGTCA  
Ala Tyr Pro Glu Glu End

226

primer  $\beta$ -4

1550

ATGCGAAATA CACATTGGTT GCGTTGTGTA TGGTGTTATT AAAGCTTGCT

# INTERNATIONAL SEARCH REPORT

International Application No

PCT/NL 91/00153

<b>I. CLASSIFICATION OF SUBJECT MATTER</b> (if several classification symbols apply, indicate all) <sup>6</sup>		
According to International Patent Classification (IPC) or to both National Classification and IPC Int.C1.5      C 12 N 15/12      C 12 Q 1/68      C 07 K 15/00 G 01 N 33/50      A 61 K 39/00		
<b>II. FIELDS SEARCHED</b>		
Minimum Documentation Searched <sup>7</sup>		
Classification System	Classification Symbols	
Int.C1.5	C 12 N      C 12 Q      C 07 K A 61 K      G 01 N	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched <sup>8</sup>		
<b>III. DOCUMENTS CONSIDERED TO BE RELEVANT<sup>9</sup></b>		
Category <sup>10</sup>	Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup>	Relevant to Claim No. <sup>13</sup>
Y	Parasitology Today, volume 6, no. 4, April 1990, M.H. Roos: "The molecular nature of benzimidazole resistance in Helminths", page 125, see page 125, column 1, line 48 - column 2, line 33; page 126, column 3, lines 44-63 ---	1-8
Y	The Journal of Cell Biology, volume 109, no. 6, December 1989, The Rockefeller University Press, M. Driscoll et al.: "Genetic and molecular analysis of a caenorhabditis elegans beta-tubulin that conveys benzimidazole sensitivity", pages 2993-3003, see the whole article ---	1-8
A	International Journal for Parasitology, volume 17, no. 3, 1987, Pergamon Journals Ltd (GB), E. Lacey et al.: "Further investigations of the primary mechanism of benzimidazole resistance in haemonchus contortus", pages 1421-1429 --- -/-	
<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p><sup>10</sup> Special categories of cited documents:</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"&amp;" document member of the same patent family</p> </div> </div>		
<b>IV. CERTIFICATION</b>		
Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report	
06-11-1991	02.12.91	
International Searching Authority	Signature of Authorized Officer	
EUROPEAN PATENT OFFICE	Nicole De Bie	

## III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
P,X	Molecular and Biological Parasitology, volume 43, 4 October 1990, Elsevier Science Publishers B.V., M.H. Roos et al.: "Molecular analysis of selection for benzimidazole resistance in the sheep parasite haemonchus contortus", pages 77-88, see the whole article -----	1-8